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Influence of chloride on the chronic toxicity of sodium nitrate to *Ceriodaphnia dubia* and *Hyalella azteca*

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Abstract While it has been well established that increasing chloride concentration in water reduces the toxicity of nitrite to freshwater species, little work has been done to investigate the effect of chloride on nitrate toxicity. We conducted acute and chronic nitrate (as sodium nitrate) toxicity tests with the cladoceran *Ceriodaphnia dubia* and the amphipod *Hyalella azteca* (chronic tests only) over a range of chloride concentrations spanning natural chloride levels found in surface waters representative of watersheds of the Great Lakes Region. Chronic nitrate toxicity test results with both crustaceans were variable, with *H. azteca* appearing to be one of the more sensitive invertebrate species tested and *C. dubia* being less sensitive. While the variability in results for *H. azteca* were to an extent related to chloride concentration in test water that was distinctly not the case for *C. dubia*. We concluded that the chloride dependent toxicity of nitrate is not universal among freshwater crustaceans. An additional sodium chloride chronic toxicity test with the US Lab strain of *H. azteca* in the present study suggested that when present as predominantly sodium chloride and with relatively low concentrations of other ions, there is a narrow range of chloride concentrations over which this strain is most fit, and within which toxicity test data are reliable.

Keywords Chronic nitrate toxicity · Acute nitrate toxicity · Chronic chloride toxicity · *Ceriodaphnia dubia* · *Hyalella azteca*

Introduction

Food and energy production, and urbanization have substantially altered the global nitrogen cycle (reviewed by Camargo and Alonso 2006). The most abundant form of anthropogenic inorganic nitrogen in freshwaters is nitrate (NO_3^-) while ammonium (NH_4^+), and nitrite (NO_2^-) tend to account for a much smaller fraction of this pool (Stanley and Maxted 2008). Nitrite is primarily thought to cause toxicity by converting oxygen-carrying blood pigments like hemoglobin and hemocyanin to forms that cannot carry oxygen like methemoglobin and methemocyanin (Camargo and Alonso 2006). It is well established that increasing chloride concentration in water reduces the toxicity of nitrite to freshwater species; this is most likely due to competitive inhibition, because in crayfish and fish gills, nitrite and chloride enter via the same route (Camargo and Alonso 2006; Jensen 1996; Harris and Coley 1991; Lewis and Morris 1986). Nitrate is thought to have a similar toxic mechanism to that of nitrite, but it is thought that organisms convert nitrate to nitrite in vivo, leading to toxicity (Cheng and Chen 2002).

Much of the published work investigating the influence of chloride on nitrite toxicity focused on fish aquaculture (Alcaraz and Espina 1994; Bartlett and Neumann 1998; Lewis and Morris 1986; Tomasso 1986; Tomasso et al. 2003) and decapods (Beitinger and Huey 1981; Gutzmer and Tmoasso 1985; Harris and Coley 1991; Kozák et al. 2005), but potential antagonistic interactions between chloride and nitrite have been investigated in other

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invertebrates like amphipods, midges, and planaria as well (Alonso and Camargo 2008; Neumann et al. 2001). For example, Alonso and Camargo (2008) demonstrated that increasing chloride concentration from 27 to 108 mg/L decreased percent mortality of the amphipod *Eulimnogammarus toletanus* exposed to 5.1 mg NO_2–N/L from ~90 to <20 % after 96 h (median lethal concentrations (LC50s) were not generated). The influence of chloride on nitrite toxicity can be substantial; in tests with the crayfish *Orconectes limosus*, increasing chloride concentration in test water from 11 to 400 mg/L increased 96 h LC50s from 4.8 to 96.6 mg NO_2–N/L (Kozák et al. 2005). Chloride has also been shown to reduce nitrite toxicity to marine shrimp (Cheng and Chen 1998).

Much less work has been done to determine if chloride affects the aquatic toxicity of nitrate. Because nitrate has a similar mechanism of uptake and toxicity to that of nitrite (Camargo and Alonso 2006), and increased chloride concentrations have also been shown to ameliorate fluoride toxicity to net-spinning caddisflies (Camargo 2004), and sulfate toxicity to the amphipod *Hyalella azteca* (Soucek 2007), one could predict that chloride might regulate nitrate toxicity as well. Soucek et al. (2015) investigated the influence of chloride on acute nitrate toxicity to two genetically distinct "strains" of the amphipod *H. azteca*, observing a strong relationship between the anions (higher chloride/lower nitrate toxicity) for what was called the "US Lab strain" by Major et al. (2013), but no relationship for the "Burlington strain". Because of the disparity between the two strains, they concluded that the chloride dependent acute toxicity of nitrate in the US lab strain, and the chloride dependent sulfate toxicity in the same strain observed by Soucek (2007) were phenomena unique to that strain, and not broader toxicological interactions. However, Scott and Crunkilton (2000), testing chronic effects of nitrate on the cladoceran *Ceriodaphnia dubia* using a water with chloride of about 2–4 mg/L, observed toxic effects at concentrations about 14 fold lower than those observed in a preliminary study conducted at the Illinois Natural History Survey using a water with about 34 mg/L chloride (DJS unpublished data). Furthermore, Tsai and Chen (2002) observed that increasing salinity decreased nitrate toxicity to the marine shrimp *Penaeus monodon*. Therefore, further work should be done to determine the extent to which chloride regulates nitrate toxicity in other freshwater species.

Few studies have been published on the chronic toxicity of nitrate to North American freshwater invertebrates. Species tested include the cladocerans *C. dubia* and *Daphnia magna* (Scott and Crunkilton 2000; *D. magna* test was sub-chronic), the amphipod *Gammarus pseudolimnaeus* (Stelzer and Joachim 2010) the Florida apple snail *Pomacea paludosa* (Corrao et al. 2006), and the mayflies *Neocloeon triangulifer* (Soucek and Dickinson 2015) and *Deleatidium* sp. (Martin and Thompson 2012). Because of the disparity in our preliminary findings with *C. dubia* and those of Scott and Crunkilton (2000), and because of the lack of effects observed at up to 128 mg N–NO_3/L for the amphipod *G. pseudolimnaeus* (Stelzer and Joachim 2010), our intent was to generate 7 and 42-d (day) chronic nitrate toxicity data with *C. dubia* and another amphipod, *H. azteca* (US lab strain), respectively. Furthermore, because of the strong relationship between chloride and nitrite toxicity, and the equivocal data on the influence of chloride on nitrate toxicity, we conducted acute and chronic toxicity tests with *C. dubia* and *H. azteca* (chronic tests only) over a range of chloride concentrations spanning natural chloride levels found in surface waters representative of watersheds of the Great Lakes Region (D. Mount, SETAC presentation 2012). Acute nitrate toxicity tests with *H. azteca* in test waters with varying chloride concentrations were previously reported in Soucek et al. (2015). Finally, because to our knowledge, no published data exist on 42-d chronic chloride toxicity to the US Lab strain of *H. azteca*, we conducted such a test to better characterize the potential interaction between chloride and nitrate. This information would provide an upper limit for chloride concentration in dilution water that when combined with previously reported minimum values (Soucek et al. 2015) could set a range of chloride concentrations over which one could expect valid chronic toxicity responses for this strain of *H. azteca.*

Methods

General culturing methods

The cladoceran, *C. dubia* was cultured according to USEPA (2002) methods, at 25 °C and a 16:8 (L:D) photoperiod. They were fed 0.5 mL of a yeast-cereal leaves-trout chow (YCT)/*Pseudokirchneriella subcapitata* (3.0×10^7 cells/mL) mixture (1:1, v.v.) daily. This was ~25 % more food than that recommended in USEPA (2002). Cladocerans were cultured in the same water in which they were tested (detailed below).

The genetic strain of *H. azteca* used for chronic toxicity testing was genetically identified as the "US Lab strain" by Major et al. (2013), and complete cytochrome oxidase subunit 1 sequences can be found at Genbank accession nos. JX446307 and JX446308 (http://www.ncbi.nlm.nih.gov/genbank/). Amphipods were cultured in a reconstituted water with a nominal hardness of 100 mg/L as $CaCO_3$, prepared according to a formula developed at the USEPA laboratory in Duluth, MN (D. Mount, Personal Communication.), which will be referred to hereafter as "culture water". To make this water, the following salt

concentrations were added to deionized water: $KHCO_3$ 10 mg/L; $NaHCO_3$ 125 mg/L; $MgSO_4$ 38 mg/L; $CaSO_4$ 40 mg/L; $CaCl_2$ 43 mg/L; NaBr 0.05 mg/L. The inclusion of NaBr provided sufficient bromide to promote good performance of this strain of *H. azteca* (Ivey and Ingersoll 2016) in review. Once salts were added to deionized water, the solution was aerated vigorously for approximately 48 h prior to use. Organisms were cultured at 25 °C, with a 16:8 (L:D) photoperiod. 25–30 individuals were held in 1-L beakers with 1000 mL of water. Nylon screen (1-mm mesh size, 44 % open area; ELKO Filtering Co., Miami, FL) was provided as substrate. At any given time three to five beakers were maintained. Organisms in each beaker were fed daily ~6 mg of dry, ground and sieved (<250 µm) Tetramin® (TetraWerke, Melle, Germany), and 5.0 mg dry weight (dw) of mixed diatom solution prepared as described in Soucek et al. (2013). Diatom species used included *Mayamaea* sp., and *Nitzschia* sp. Both genera were obtained from Carolina Biological (Burlington, NC), sold as *Navicula* sp. and *Synedra* sp., respectively. Genus level identities were taxonomically confirmed by an expert (S. Decelles) at USEPA-ORD, Cincinnati, OH. Culture water was changed and screens were cleaned twice weekly; young were counted and collected at that time. Upon collection, young were transferred to a 23 °C environmental chamber for acclimation to test temperature. During this acclimation period, each beaker of *H. azteca* was fed ~3 mg of dry, ground and sieved (<250 µm) Tetramin, and 5.0 mg (dw) of mixed diatom solution daily. They were also slowly acclimated to test water (50 % change over with test water per day) over the course of a week prior to testing.

Test waters

Overall, seven different reconstituted waters ("test waters" hereafter) were formulated for acute and chronic sodium nitrate toxicity testing with nominal chloride concentrations of 5, 10, 25, 50, 75, 100, and 200 mg Cl/L (Table S1). Test water recipes were based on another reconstituted water formula developed at the USEPA research laboratory in Duluth, MN (D. Mount, Personal Communication.), having a nominal hardness of 90 mg/L. Test waters differed from culture water primarily in that $CaCO_3$ was the main source of carbonate rather than $NaHCO_3$. All test waters had the following salt concentrations (Reagent or certified ACS grade salts) added to deionized water: 3.6 mg/L of KCl, 18 mg/L of $NaHCO_3$, 60 mg/L of $MgSO_4{\cdot}7H_2O$, 8.3 mg/L of $MgCl_2{\cdot}6H_2O$, 61.6 mg/L of $CaCO_3$, and 0.05 mg/L of NaBr. Different chloride concentrations were achieved by adding 0.7, 8.9, 17.3, 33.8, 74.9, 116.0, 157.5, and 322.5 mg/L of NaCl, to the Cl = 5, Cl = 10, Cl = 15, Cl = 25, Cl = 50, Cl = 75, Cl = 100, and Cl = 200 test waters, respectively. Thus the only difference in ionic composition among the various test waters the amount of sodium and chloride. Carbon dioxide gas (99.9 % CO_2) was bubbled through the solution to dissolve $CaCO_3$, followed by natural air to bring the pH back to ~7.6 for testing.

For acute nitrate toxicity tests with *C. dubia,* the 5, 10, 25, 50, 75, 100, and 200 mg Cl/L test waters were used (Table S1). Because of high inter-test variability, five separate tests were conducted using the Cl = 5 mg/L water, and four were conducted in the Cl = 100 mg/L water. Only two tests were conducted in the chloride = 200 mg/L water. Three tests were conducted in all other test waters.

One chronic nitrate toxicity test was conducted with *C. dubia* in each of the Cl = 10, 25, 50, and 100 mg/L test waters (Table S1).

Chronic toxicity tests with *H. azteca* were conducted in the Cl = 10, 25, and 100 mg/L waters (Table S1). Fewer test waters were used in the *H. azteca* testing because of the longer duration of the test. Results of *H. azteca* acute nitrate toxicity tests in these same test waters were previously reported in Soucek et al. (2015).

In addition to nitrate chronic toxicity tests at three different chloride concentrations, we conducted a 42-d NaCl toxicity test with *H. azteca* (US Lab strain). A previous study (Soucek et al. 2015) investigated the influence of lower concentrations (5 to 100 mg/L) of chloride on survival, growth and reproduction of this species, but no information existed to our knowledge on at what point chloride becomes toxic in 42-d tests. This 42-d sodium chloride chronic test was conducted using the "culture water" described above (Table S1).

Acute toxicity testing procedures

For *C. dubia,* static, non-renewal, acute toxicity tests were conducted according to guidelines detailed in American Society for Testing and Materials (ASTM 2002a) E729-96 (Table S2). The nitrate source for acute toxicity tests was sodium nitrate ($NaNO_3$, reagent grade, CAS# 7631-99-4, Fisher Scientific, Itasca, IL). For all tests, treatments were comprised of a 50 % dilution series (nominal concentrations of 1129, 565, 282 141, 71, and 0 (control) mg $N–NO_3$/L) with test waters described above being used as both the diluents and controls. Four replicates were tested per concentration, and five organisms were added to each replicate. Test duration was 48 h. None of the acute tests were aerated or fed. Tests were conducted at 25 ± 1 °C with a 16:8 (L:D) photoperiod. Test chambers were 50 mL glass beakers. Test organisms were <24 h old at the beginning of the test. Percent survival in each replicate was recorded every 24 h and at the end of the exposure period.

Chronic toxicity testing procedures

Ceriodaphnia dubia

For *C. dubia,* 7-day, three-brood chronic bioassays were conducted according to guidelines described in (ASTM 2002b) E 1295-01 (Table S3). Briefly, one <24-h-old neonate was placed in each of ten replicate 50 mL beakers for each of six nitrate concentrations, including a control (no nitrate added). For all tests, treatments were comprised of a 50 % dilution series (nominal concentrations of 400, 200, 100, 50, 25 and 0 (control) mg N–NO_3/L). Each test organism was fed at a rate identical to that used for culturing (USEPA 2002), and test solutions were renewed daily. Neonates produced by the first generation test organisms were counted daily and the test duration was 7 days unless 60 % of control organisms produced three broods by day six. Endpoints included survival of first generation *C. dubia*, and the number of young produced by each first generation organism.

Hyalella azteca

Water-only, 42-d, renewal, nitrate toxicity tests were performed using recommendations detailed in the USEPA sediment toxicity testing guidelines (USEPA 2000), but with modifications (Table S4). For all tests, treatments were comprised of a 50 % dilution series (nominal concentrations of 200, 100, 50, 25, 12.5 and 0 (control) mg N–NO_3/L). Test chambers were 300 mL, high-form glass beakers with 200 mL of test water (described above) added to each beaker. Approximately 3.5 × 4 cm pieces of nylon screen (1 mm mesh size, 44 % open area; ELKO Filtering Co., Miami, FL) were provided to each beaker as substrate. Tests were performed at 23 °C under a 16:8 L:D photoperiod without aeration. Organisms were 7 to 9 days old at the beginning of the tests, and 10 individuals were added to each of five replicate test chambers per treatment. Complete water renewals were performed 3 times weekly (MWF), with test organisms being transferred to clean weigh-boats containing test water while test chambers were cleaned. Substrates were replaced weekly. During the test, organisms were fed daily ground and sieved (<250 µm) Tetramin and mixed diatom solution described above in "General culturing methods" section. Diatoms were added to beakers at a rate of 1 mg (dw) per day throughout the test. Tetramin feeding rate was as follows: 1 mg (dw) daily during week one, 1.25 mg daily during weeks two and three, and 2.5 mg daily during weeks four through six. After the first appearance of mating pairs, contents of each test chamber were carefully searched for young on water renewal days. On test day 42, adult amphipods were placed in a small amount of 95 % ethanol so they could be examined under a dissecting scope to determine sex (males have an enlarged and modified second gnathopod). They were then immediately transferred to aluminum weigh pans and dried in an oven (60–70 °C) for at least 48 h before they were weighed to the nearest 0.001 mg using a Cahn C-35 microbalance. Based on subsamples of organisms used to initiate the tests, average starting weights for the 42-d tests were 0.018, 0.016, and 0.038 mg dw per individual, for the Cl = 10, Cl = 25, and Cl = 100 mg/L tests, respectively. Endpoints included percent survival, growth (average increase in mean dry weight per individual), biomass (combined mass of surviving adults), and # of young per surviving female.

For the chronic NaCl toxicity test with *H. azteca* (US Lab strain), we used the same 42-d test methods as described above with "culture water" as the diluent and control, and nominal chloride (as sodium chloride) concentrations of 27 (control), 213, 399, 771, 1514, and 3000 mg Cl/L. The highest concentration was similar to the 96-h LC50 reported using the same dilution water in Soucek et al. (2013).

Water chemistry

For both chronic and acute tests, standard water quality measurements were made on new and old test solutions (for each water change for the chronic tests), including temperature, pH, conductivity, dissolved oxygen, alkalinity, and hardness. Alkalinity and hardness were measured in new water only and dissolved oxygen in old water only. The remaining parameters were measured in both old and new water. The pH measurements were made using an Accumet® (Fisher Scientific, Pittsburgh, PA, USA) model AB15 pH meter equipped with an Accumet® gel-filled combination electrode (accuracy < ± 0.05 pH at 25 °C). Dissolved oxygen was measured in "out water" after water renewals using an air-calibrated Yellow Springs Instruments (RDP, Dayton, OH, USA) model 55 m. Conductivity measurements were made using a Mettler Toledo® (Fisher Scientific, Pittsburgh, PA, USA) model MC226 conductivity/TDS meter. Alkalinity and hardness were measured by titration as described in (American Public Health Association 2005). Water samples from each treatment were submitted to the IL State Water Survey analytical laboratory for confirmation of chloride and nitrate (reported as N–NO_3) concentrations using ion chromatography. We did not measure ammonia, but in other tests with nitrate concentrations ranging up to 200 mg N–NO_3/L (data not included in this manuscript), ammonia concentrations remained very low (<0.03–0.09 mg N–NH_4) so we were not concerned about conversion of nitrate to ammonia in our water-only test system. Measured water quality parameters were similar to

expected values, as were measured toxicant concentrations (Table S5). For the nitrate acute and chronic tests, chloride concentrations in test waters were similar to expected values with mean percent measured/nominal concentrations ranging from 97 to 99 %.

Statistical analysis

For acute toxicity tests, median lethal concentrations (LC50s) were calculated using the trimmed Spearman-Karber method (Hamilton et al. 1977). For chronic toxicity tests, differences among treatments in survival for both species were analyzed statistically using Fisher's exact test, and means for the remaining endpoints were compared using ANOVA with post hoc pairwise comparisons conducted using either Dunnett's or Steel's Many-One test depending on whether or not data were normal and variances were homogenous. The 20 and 50 % effective concentrations (EC20s and EC50s) were calculated using TRAP® software (R. Erickson, USEPA Duluth). Relationships between chloride in dilution water and acute and chronic nitrate toxicity endpoints were analyzed using linear regression analysis.

Results

Acute toxicity testing

Ceriodaphnia dubia

Most (18 of 23) 48-h nitrate toxicity tests at varying chloride concentrations with *C. dubia* had 100 % control survival; three had 95 % control survival, and two tests had 90 % control survival (Table S6). The measured 48-h LC50s spanned slightly greater than two fold, with the lowest being 369 mg N–NO_3/L at nominal chloride of 100 mg/L and the highest being 799 mg N–NO_3/L at nominal chloride of 25 mg/L (Table S6). Chloride concentration in dilution water was not significantly correlated with nitrate LC50 ($R^2 = 0.0307$, $p = 0.424$; Fig. 1).

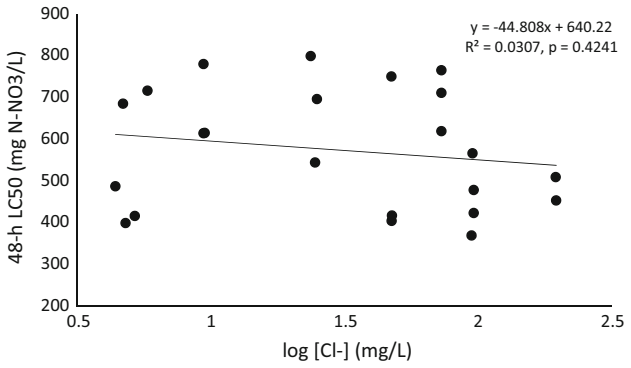

Fig. 1 Relationship between chloride in dilution water and acute nitrate (as $NaNO_3$) toxicity to the cladoceran *C. dubia*. Confidence limits for individual LC50s are provided in supplemental Table 3

Chronic toxicity testing

Ceriodaphnia dubia nitrate chronic tests

In all four chronic nitrate toxicity tests at different chloride concentrations, control survival was 100 % and reproduction values met minimum test acceptability criteria of ≥15 young produced per surviving female (Table S7). In fact, in all four chronic tests, only one first-generation individual died in any of the treatments (405 mg N–NO_3/L in the Cl = 10 test). This was somewhat surprising because some of the 48 h LC50s for this species were near the highest test concentrations in the chronic tests. It is likely that the addition of food in the chronic tests account for this decreased sensitivity. Control reproduction ranged from 15.3 ± 5.1 (Cl = 10) to 39.6 ± 2.3 (Cl = 100) young per female (Table S7), and dose–response curves did not vary in accordance with chloride concentration in dilution water (Fig. 2). Because of the lack of survival response, all reported EC20s are for reproduction. While EC20s ranged from 80 (Cl = 50) to 263 (Cl = 10) mg N–NO_3/L (an approximately three fold difference; Table S7), there was no apparent relationship between chloride concentration in dilution water, and chronic nitrate toxicity to *C. dubia* whether in terms of EC20 ($R^2 = 0.028$, $p = 0.832$) or acute to chronic ratio (ACR; $R^2 = 0.079$, $p = 0.721$). Acute to chronic ratios based on EC20s ranged from 2.5 to 7.4 (Table 1).

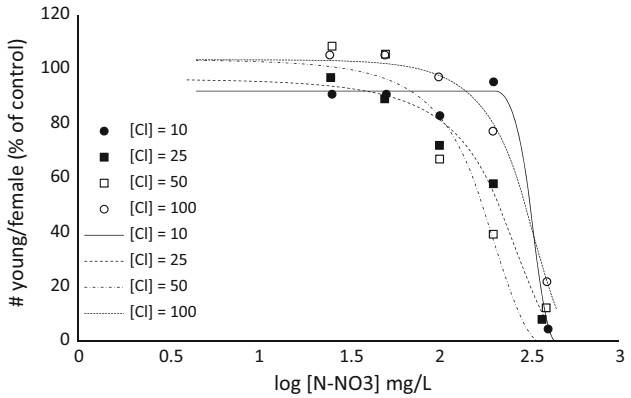

Fig. 2 Influence of nitrate (as $NaNO_3$) on reproduction of the cladoceran *C. dubia* in 7-days chronic tests conducted in dilution waters with different chloride concentrations. *Lines* shown are predicted from TRAP® software (R. Erickson, USEPA Duluth) used to calculate effective concentrations (ECx). Control values not shown because data are expressed as percent of control. Actual reproduction data are provided in Supplemental Table 5

Table 1 Nitrate (as sodium nitrate) acute and chronic values for *H. azteca* and *C. dubia* determined in dilution waters with different chloride concentrations

Measured [Cl]	48 h LC50[a]	EC20	EC50 (ACR)
C. dubia			
10.1 mg/L	665	263 (2.5)	306 (2.2)
24.9 mg/L	671	91 (7.4)	183 (3.7)
48.7 mg/L	502	80 (6.3)	153 (3.3)
96.6 mg/L	453	177 (2.6)	271 (1.7)
Measured [Cl]	96 h LC50[b]	EC20[c] (ACR[d])	EC50 (ACR)
H. azteca			
9.9 mg/L	210	11 (19.1)	32 (6.6)
24.6 mg/L	516	19 (27.2)	62 (8.3)
97.6 mg/L	736	25 (29.4)	86 (8.6)

LC50 median lethal concentration; *EC20* 20 % effective concentration; *ACR* acute to chronic ratio. All *LC50*, *EC20* and *EC50* concentrations in mg $N–NO_3^-$/L

[a] *C. dubia* LC50s are geometric means of acute toxicity tests at given [Cl]

[b] *H. azteca* LC50s are geometric means of tests at given [Cl] reported in Soucek et al. (2015)

[c] EC20s and EC50s shown are lowest (most sensitive) values obtained for each test

[d] ACRs calculated as LC50/EC20 or LC50/EC50

Hyalella azteca nitrate chronic tests

In all three chronic nitrate toxicity tests at different chloride concentrations, controls performed well in terms of survival, reproduction and growth. Control survival ranged from 88 (Cl = 10 and 25) to 92 % (Cl = 100), reproduction ranged from 10.0 ± 5.4 (Cl = 25) to 25.6 ± 4.0 (Cl = 100) young per female, and growth ranged from 0.800 ± 0.045 (Cl = 10) to 1.281 ± 0.145 (Cl = 100) mg dry weight (Table 2). In all cases, survival, reproduction, and growth values for controls surpassed newly proposed test acceptability criteria for sediment toxicity tests with this species (Ivey et al. 2016).

No single endpoint was the most sensitive in all three tests, with EC20s being the most sensitive (lowest) for biomass, growth, and reproduction at Cl = 10, 25, and 100, respectively (Table 2). For the Cl = 10 test, EC20s ranged from 11 (biomass) to 50 (growth) mg $N–NO_3$/L, while at Cl = 25, EC20s ranged from 19 (growth) to 121 (survival) mg $N–NO_3$/L, and at Cl = 100 they ranged from 25 (young/female) to 56 mg $N–NO_3$/L (growth; no EC20 was calculated for survival in this test). Taking the lowest EC20 for each test, they ranged from 11 (Cl = 10) to 25 (Cl = 100) mg $N–NO_3$/L, just over a two fold increase. The calculated acute to chronic ratios (ACR = LC50/EC20) increased by less than two fold over this range, from 19.1 at Cl = 10 to 29.4 at Cl = 100 (Table 1). The overall geometric mean species ACR (based on EC20s) was 24.8. While there was a clear increase in the most sensitive metric for both EC20 and ACR with increasing chloride in dilution water (Table 1), there were too few data points to generate a statistically significant relationship.

Hyalella azteca chloride chronic test

In the chronic chloride toxicity test, controls performed well in terms of survival (92 %), reproduction (28.9 young per surviving female), growth (1.159 mg dry weight per individual), and biomass (10.940 mg dry weight) (Table 3). Dose dependent responses were observed for all endpoints with biomass (EC20 = 183 mg Cl/L) being the most sensitive and reproduction (EC20 = 735 mg Cl/L) being the least sensitive. Acute to chronic ratios were calculated using the mean LC50 (3.032 mg Cl/L) reported using this dilution water by Soucek et al. (2013), and ranged from 4.1 (reproduction) to 16.6 (biomass) (Table 3).

Discussion

While several of the *C. dubia* LC50s from the present study were similar to those reported in Scott and Crunkilton (2000) for the same species (374 mg $N–NO_3$/L in each of two separate tests), the overall species mean acute value for the present study was higher at 558 mg $N–NO_3$/L (Table S6). The *C. dubia* nitrate LC50s in the present study spanned slightly more than a two fold range. This level of variability was much less than the nearly ten fold range observed for the US Lab strain of *H. azteca* in a previous study (Soucek et al. 2015) over a similar range of chloride concentrations (5–100 mg/L). However, Soucek et al. (2015) concluded that for that particular strain of *H. azteca*, toxicity data reported using dilution waters with less than 15–20 mg Cl/L should be used with caution. If only LC50s for *H. azteca* from the Soucek et al. (2015) study generated in waters with Cl ≥15 mg/L are used, the range of LC50s (~3.3 fold) is more similar to that of *C. dubia* in the present study. However, unlike the US Lab strain of *H. azteca*, *C. dubia* nitrate LC50s in the present study were not significantly correlated with chloride concentration in the dilution water. In fact, for *C. dubia*, variability among individual tests conducted at the same chloride concentration was nearly as great as the variability observed over the entire range of test conditions. For example, LC50s in the Cl = 5 mg/L water varied from 399 to 716 mg $N–NO_3$/L. Interestingly, the total range of nitrate LC50s reported for the "Burlington strain" of *H. azteca* was only ~1.3-fold across all chloride concentrations (Soucek et al. 2015), and while the US Lab strain LC50s co-varied

Table 2 42-d chronic nitrate (as sodium nitrate) toxicity data for *H. azteca* in dilution waters with varying chloride concentrations

[N–NO_3^-][a] (mg/L)	Survival (%)	# Young per female	Growth (mg dw)	Biomass (mg dw)
Cl = 10 mg/L				
0.03 (control)	88 ± 7 A	12.7 ± 2.3 A	0.800 ± 0.045A	7.173 ± 0.438 A
11.7	66 ± 21 A	13.6 ± 6.9 A	0.726 ± 0.107 A	4.918 ± 2.038 B
24	60 ± 17 B	12.8 ± 3.5 A	0.743 ± 0.077 A	4.491 ± 1.124 B
48	52 ± 12 B	10.5 ± 6.8 A	0.670 ± 0.087 A	3.552 ± 0.984 B
97	20 ± 6 B	0 ± 0 B	0.323 ± 0.179 B	0.772 ± 0.587 B
195	6 ± 5 B	0 ± 0 B	0.074 ± 0.023 B	0.092 ± 0.028 B
EC20 (95 % CI)	14 (9–23)	48 (37–62)	50 (38–65)	11 (6.1–19)
EC50 (95 % CI)	41 (32–54)	58 (0.1–41057)	84 (71–100)	32 (24–43)
Cl = 25 mg/L				
0.02 (control)	88 ± 12 A	10.0 ± 5.4 A	1.096 ± 0.077 A	9.706 ± 0.801 A
12	90 ± 9 A	11.1 ± 4.6 A	0.882 ± 0.152 B	7.887 ± 1.771 A
24	90 ± 13 A	11.0 ± 7.0 A	0.867 ± 0.166 B	7.907 ± 1.619 A
49	84 ± 14 A	7.3 ± 3.4 A	0.686 ± 0.075 B	5.909 ± 1.229 B
98	78 ± 4 A	2.7 ± 1.7AB	0.475 ± 0.097 B	3.825 ± 0.779 B
197	56 ± 8 B	0 ± 0 B	0.146 ± 0.017 B	0.904 ± 0.126 B
EC20 (95 % CI)	121 (81–179)	43 (22–84)	19 (13–27)	22 (15–32)
EC50 (95 % CI)	>197	66 (42–104)	64 (51–79)	62 (49–78)
Cl = 100 mg/L				
0.02 (control)	92 ± 7 A	25.6 ± 4.0 A	1.281 ± 0.145 A	12.043 ± 0.678 A
12	88 ± 12 A	25.2 ± 7.4 A	1.193 ± 0.151 A	10.943 ± 2.333 A
24	82 ± 17 A	17.5 ± 5.1 A	1.098 ± 0.077 A	9.265 ± 1.921 A
48	82 ± 7 A	19.5 ± 4.5 A	1.058 ± 0.148 A	8.889 ± 0.508 B
97	72 ± 10 A	9.8 ± 3.1 B	1.040 ± 0.207 A	7.948 ± 2.609 B
195	72 ± 12 A	8.2 ± 2.9 B	0.698 ± 0.130 B	5.426 ± 1.793 B
EC20 (95 % CI)	nc	25 (14–47)	56 (35–88)	28 (15–54)
EC50 (95 % CI)	nc	86 (57–129)	>195	176 (97–319)

Within endpoint columns, means followed by different capital letters are significantly different ($p < 0.05$)

nc not calculated (due to insufficient effect), *EC*20 20 % effect concentration, *dw* dry weight, *CI* confidence interval

[a] Mean measured N–NO_3^- concentrations are shown for all tests

Table 3 42-d chronic chloride (as sodium chloride) toxicity data for *H. azteca*

[CL^-][a] (mg/L)	Survival (%)	# Young per female	Growth (mg dw)	biomass (mg dw)
27 (control)	92 ± 7 A	28.9 ± 12.83 A	1.159 ± 0.077 A	10.940 ± 1.351 A
216	88 ± 13 A	28.1 ± 8.4 A	1.089 ± 0.124 A	9.028 ± 0.975 A
402	42 ± 15 B	42.4 ± 13.9 A	0.845 ± 0.159 A	3.497 ± 1.018 B
778	42 ± 20 B	23.4 ± 14.5 A	0.544 ± 0.202 A	2.554 ± 1.475 B
1529	14 ± 14 B	10.0 ± 4.5 A	0.488 ± 0.270 B	0.708 ± 0.704 B
3090	0 ± 0 B	0 ± 0 B	na	na
EC20 (95 % CI)	236 (173–321)	735 (364–1486)	313 (192–509)	183 (130–259)
EC50 (95 % CI)	560 (464–677)	1109 (649–1895)	910 (651–1273)	358 (300–427)
ACR[b]	12.8	4.1	9.7	16.6

Within endpoint columns, means followed by different capital letters are significantly different ($p < 0.05$)

na not applicable, *EC*20 20 % effect concentration, *dw* dry weight, *CI* confidence interval, *ACR* acute to chronic ratio

[a] Mean measured Cl^- concentrations are shown

[b] ACR calculated as median lethal concentration (LC50) divided by EC20 using LC50 (3,032 mg Cl/L) reported in Soucek et al. (2013) using the same dilution water

strongly with chloride concentration in dilution water, a maximum of 1.5 fold range in LC50s was observed among tests conducted in a given dilution water (Soucek et al. 2015).

While 48-h nitrate LC50s for *C. dubia* were somewhat variable, even the lowest observed values would only place the species at approximately the median in terms of sensitivity of species reported in the literature. Species observed to be more acutely sensitive than *C. dubia* (based on our overall geometric mean of 558 mg N–NO_3/L) include amphipods (Camargo et al. 2005), caddisflies (Camargo and Ward 1992; Camargo et al. 2005), a mayfly (Soucek and Dickinson 2015), two freshwater bivalves (Soucek and Dickinson 2012), the cladoceran *D. magna* (Scott and Crunkilton 2000) and a stonefly (Soucek and Dickinson 2012). It is complicated to compare the nitrate sensitivity of *C. dubia* to that of *H. azteca* both because of the fact that the latter is now considered a complex of cryptic species (Duan et al. 1997; Hogg et al. 1998; McPeek and Wellborn 1998), and because of the chloride dependent sensitivity of at least one genetic strain (Soucek et al. 2015). The Burlington strain of *H. azteca* was more sensitive to nitrate than *C. dubia* with a mean LC50 of 387 mg N–NO_3/L (Soucek et al. 2015), and including only data produced in dilution waters with Cl $\geq$15 mg/L as discussed above, the US Lab strain of *H. azteca* is similar in sensitivity (mean of 526 mg N–NO_3/L; Soucek et al. 2015).

As was the case with acute tests, the most sensitive nitrate chronic values (EC20s) for *H. azteca* were correlated with dilution water chloride concentration (lower chloride resulted in lower EC20s) while those for *C. dubia* were not. In the case of *C. dubia*, the EC20s (all for reproduction) were variable, with an approximately three fold difference between the lowest and highest value over the range of dilution waters, but the two lowest EC20s were in the middle two chloride levels, with the highest being in the Cl = 10 and Cl = 100 waters. For *H. azteca*, the lowest EC20 for each dilution water was correlated with chloride concentration, but there were four different endpoints evaluated for each test and the variability among them was large in some cases. For example, in the Cl = 10 test, there was a ~five fold range of EC20s over the four endpoints, and for the Cl = 25 test, there was a six fold range (Table 2). The chronic values in the Cl = 100 test were less variable, with only a two fold range. Taking the geometric mean of all EC20s for each dilution water produced means of 25, 38, and 34 mg N–NO_3/L for the Cl = 10, 25, and 100 mg/L waters, respectively, apparently eliminating the correlation with chloride.

In addition to the magnitude of variability in chronic values for the *H. azteca* tests, the most sensitive endpoint varied from test to test, with biomass being most sensitive in the Cl = 10 test, and growth and reproduction being most sensitive in the Cl = 25 and Cl = 100 tests, respectively. Of additional note is the overall magnitude of the ACRs for *H. azteca*, with values of 19.1, 27.2, and 29.4 for the three dilution waters, compared to ACRs ranging from 2.5 to 7.4 for *C. dubia* in the present study. Because of the variability among EC20s in the *H. azteca* tests, we also calculated EC50s to explore whether they would produce more consistent chronic values. The EC50s only varied among endpoints by ~2.5, 3, and 2.3-fold within the Cl = 10, 25, and 100 mg/L tests (compared to the above stated 5, 6, and two fold ranges in EC20s). In addition, we calculated coefficients of variation (CV) for EC20s and EC50s within a given test (dilution water), and CVs for EC20s ranged from 41 (Cl = 100) to 108 (Cl = 25), whereas CVs for EC50s ranged from 33 (Cl = 100) to 68 (Cl = 25). Finally, ACRs calculated using EC50s were more similar to those produced for *C. dubia* (using EC20s), ranging from 6.6 to 8.6. It is unclear why so much variability was observed in the *H. azteca* chronic values, but it may be related to the intimate relationship this US lab strain appears to have with chloride in dilution water (Soucek et al. 2015). It should be further noted (as discussed above) that based on the recommendations of Soucek et al. (2015), the *H. azteca* nitrate chronic toxicity test conducted with the Cl = 10 test water in the present study should be used with caution.

To our knowledge, there is a relative paucity of information in the literature on long-term chronic toxicity of nitrate to freshwater invertebrates. Chronic data have been reported for two mayfly species with strikingly similar results. A full-life chronic test with the baetid *N. triangulifer* produced a mean EC20 of 37 mg N–NO_3/L (Soucek and Dickinson 2015), and Martin and Thompson (2012) reported a 20-days threshold effect concentration (TEC; equivalent to maximum allowable toxicant concentration (MATC)) of 35 mg N–NO_3/L for the leptophlebiid mayfly *Deleatidium* sp. in a test using early stage larvae. Other taxa have been observed to be less sensitive. A mean EC50 of 560 mg N–NO_3/L was reported for juvenile Florida apple snails (*P. paludosa*) in a 14-days test (Corrao et al. 2006), and Stelzer and Joachim (2010) observed no effects on mortality, growth, egestion rates, and molting of the amphipod *G. pseudolimnaeus* at N–NO_3 concentrations up to 128 mg/L. Scott and Crunkilton (2000) previously tested the cladoceran *C. dubia* and produced an average 7-days MATC of 22 mg N–NO_3/L. This value is about six fold lower than the mean EC20 generated for this species in the present study. Furthermore, the ACRs for *C. dubia* generated in the present study were substantially lower than the mean ACR of 17 reported by Scott and Crunkilton (2000). A potential explanation for this is that the Scott and Crunkilton (2000) tests were

conducted in USEPA (2002) "hard" reconstituted water, which has ion ratios not frequently seen in natural waters (D. Mount, SETAC presentation 2012), particularly for chloride and sulfate, whereas the current tests were conducted in waters designed to better mimic natural ionic compositions. Combining the mean MATC from Scott and Crunkilton (2000) with the mean *C. dubia* EC20 from the present study results in a species mean chronic value of 55 mg N–NO_3/L. If the geometric mean of the lowest EC20 for each *H. azteca* test in the present study is used (22 mg N–NO_3/L), that species (strain) would be considered the most sensitive invertebrate, followed by *Deleatidium*, *N. trianguifer, C. dubia, P. paludosa,* and *G. pseudolimnaeus.*

Several vertebrates have been tested chronically for nitrate toxicity as well. A mean EC20 of 62 mg N–NO_3/L was generated for the fathead minnow *Pimephales promelas* (USEPA 2010). Schuytema and Nebeker (1999) conducted a 16-days test with juvenile red-legged frogs (*Rana aurora*) and obtained MATCs of 166 (wet weight) and <29.1 mg N–NO_3/L (length), although in the latter case, the difference in lengths among the Lowest Observed Effect Level and the No Observed Effect level treatments was only 2.8 %. Finally, McGurk et al. (2006) generated chronic data in 126 and 146-days tests with the lake whitefish *Coregonus clupeaformis* and the lake trout *Salvelinus namaycush,* respectively. The EC50 for the whitefish was 64.4 mg N–NO_3/L, and for the lake trout EC50s of 189.6 and 196.1 mg N–NO_3/L were produced. In the case of the lake trout, an MATC of 3.2 N–NO_3/L was also generated for the endpoints of weight and developmental delay. No EC50s were generated for either of those endpoints.

The results of our 42-d sodium chloride toxicity test with the US Lab strain of *H. azteca* indicated greater sensitivity to chloride than was expected given the relatively high 96-h LC50 in the same dilution water (3032 mg Cl/L (Soucek et al. 2013)), and the fact that this species is considered to be euryhaline (USEPA 2000). Ingersoll et al. (1992) reported the ability to culture and conduct toxicity tests with this species at salinities up to 15 g/L, and Werner et al. (2010) used this species to test the toxicity of field-collected waters with widely ranging salinities from the Northern Sacramento-San Joaquin Estuary in California, USA. However, in those studies, either artificial or natural seawaters were used, which in addition to high concentrations of sodium and chloride, would also have increased calcium and magnesium concentrations compared to those present in our reconstituted freshwater. It is well known that the ionic composition of a water with high salt concentrations determines its toxicity (Mount et al. 1997), and perhaps *H. azteca* can tolerate high salt concentrations when multiple cations are present, but is sensitive when exposed only to NaCl (with relatively low Ca and Mg present). Benbow and Merritt (2004) reported *H. azteca* to be tolerant of road salt in 15-days exposures, but they used field collected organisms so it is difficult to determine which of up to five or six genetically distinct "species" or strains they used (Major et al. 2013). More similar to our findings, Bartlett et al. (2012) reported a 28-days growth EC25 of 420 mg Cl/L in water-only sodium chloride exposures. They used the "Burlington strain" of *H. azteca* (A. Bartlett Pers. Comm), which was more acutely sensitive to sodium chloride than the US Lab strain in a previous study by Soucek et al. (2013). Elphick et al. (2011) generated a 28-days IC25 of 1705 mg Cl/L with the US Lab strain of *H. azteca*, but the test was conducted over rinsed, beach-collected sand supplemented with peat, so it is difficult to make comparisons between this test and ours given the potential impact of the different testing conditions. We are unaware of other 42-d chronic sodium chloride data generated with this strain of *H. azteca* under similar test conditions, but our results indicate a relatively narrow range of chloride concentrations over which the strain performs optimally: between 15 and 20 mg/L (Soucek et al. 2015) and ~200 mg/L (present study). Further study would help to validate this conclusion. However, we can also conclude that the nitrate chronic toxicity data from the Cl = 25 and 100 mg/L test waters in the present study are valid since the range of chloride concentrations in test waters fell below this upper limit.

Conclusion

In summary, chronic nitrate toxicity test results with both crustaceans in the present study were quite variable, with *H. azteca* appearing to be one of the more sensitive invertebrate species tested, and *C. dubia* perhaps not being as sensitive as previously reported (Scott and Crunkilton 2000). While the chronic results for *H. azteca* (US Lab strain) were to an extent related to chloride concentration in test water, that was distinctly not the case for *C. dubia.* Although Tsai and Chen (2002) observed that increasing salinity decreased nitrate toxicity to the marine shrimp *P. monodon*, and Soucek et al. (2015) observed a strong chloride dependence of acute nitrate toxicity to the US Lab strain of *H. azteca*, the results from Soucek et al. (2015) with the Burlington strain of *H. azteca* and with *C. dubia* in the present study indicate that chloride dependent nitrate sensitivity is not universal among freshwater crustaceans. Finally, the US Lab strain of *H. azteca* appears to regulate chloride ions differently than most freshwater species tested. When present as predominantly sodium chloride and with relatively low concentrations of other ions, there is a narrow range of chloride concentrations over which this

strain is most fit, and within which toxicity test data are reliable.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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